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Brefeldin A Acts to Stabilize an Abortive ARF–GDP–Sec7 Domain Protein Complex: Involvement of Specific Residues of the Sec7 Domain

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Summary

We demonstrate that the major in vivo targets of brefeldin A (BFA) in the secretory pathway of budding yeast are the three members of the Sec7 domain family of ARF exchange factors: Gea1p and Gea2p (functionally interchangeable) and Sec7p. Specific residues within the Sec7 domain are important for BFA inhibition of ARF exchange activity, since mutations in these residues of Gea1p (sensitive to BFA) and of ARNO (resistant to BFA) reverse the sensitivity of each to BFA in vivo and in vitro. We show that the target of BFA inhibition of ARF exchange activity is an ARF–GDP–Sec7 domain protein complex, and that BFA acts to stabilize this complex to a greater extent for a BFA-sensitive Sec7 domain than for a resistant one.

Introduction

Brefeldin A (BFA) is a potent inhibitor of protein secretion in eukaryotic cells (Takatsuki and Tamura, 1985; Klausner et al., 1992). BFA is a hydrophobic compound first described in 1958 (Singleton et al., 1958), which was demonstrated thirty years later to have dramatic effects on the structure and function of intracellular organelles, particularly the Golgi apparatus (Hunziker et al., 1992; Klausner et al., 1992). This discovery led to intense interest in the drug both for understanding the mechanisms underlying the organization of intracellular compartments and as a tool to specifically inhibit the functioning of the secretory pathway in eukaryotic cells. The structure of the Golgi complex is severely perturbed after only a few minutes of BFA treatment, which typically fuses with the endoplasmic reticulum (ER) after 10 min in the presence of the drug, leading to a complete block of protein transport out of the fused ER–Golgi system (Lippincott-Schwartz et al., 1989). BFA inhibits early steps in ER–Golgi transport in the budding yeast *Saccharomyces cerevisiae* (Graham et al., 1993), and, in this organism as well, dramatic effects on Golgi structure are observed within minutes of BFA treatment (Rambourg et al., 1995).

A major breakthrough in understanding the molecular action of BFA came with the discovery that it specifically inhibits a Golgi-associated guanine nucleotide exchange activity for the small GTP-binding protein ADP-ribosylation factor 1, or ARF1 (Donaldson et al., 1992; Helms and Rothman, 1992). ARF proteins exist in both soluble, inactive GDP-bound and active membrane-associated GTP-bound forms (Donaldson et al., 1991; Serafini et al., 1991). ARF proteins have been implicated in a number of different protein transport steps in both yeast and mammalian cells, including trafficking between the ER and the Golgi apparatus, and function to recruit COPI and AP-1/clathrin coat protein complexes to Golgi membranes (Boman and Kahn, 1995; Gaynor et al., 1998).

We identified a pair of exchange factors for ARF in *Saccharomyces cerevisiae*, Gea1p and Gea2p (Peyroche et al., 1996). These proteins are 50% identical and are functionally redundant, that is, deletion of one or the other in yeast has no detectable phenotype, but the double-deletion strain is inviable. Purified Gea1p has ARF exchange activity in vitro that is sensitive to BFA (Peyroche et al., 1996). Gea1p (160 kDa) and Gea2p (166 kDa) each contain a centrally located region of about 200 amino acids also found in yeast Sec7p, the "Sec7 domain" (see below). Homologs of the yeast Gea1/2 proteins have been identified in *Homo sapiens* (GBF1, Mansour et al., 1998), *Caenorhabditis elegans* (GenBank accession number Z81475), and *Arabidopsis thaliana* (GNOM/Emb30p, Shevell et al., 1994), and range in size from 160 to 210 kDa. Yeast Sec7p homologs (180–230 kDa) have been found in *Bos taurus* (p200) (Morinaga et al., 1997), *A. thaliana* (GenBank accession number AL022604), and *Schizosaccharomyces pombe* (GenBank accession number Z98602). Both p200 and the Sec7 domain of yeast Sec7p have ARF exchange activity in vitro that is inhibited by BFA (Morinaga et al., 1997; Sata et al., 1998). These results and the fact that both Gea1/2p and Golgi-localized Sec7p are required for ER-through-Golgi transport in yeast (Franzusoff et al., 1991; Peyroche et al., 1996) support the idea that members of the Gea1/2p and Sec7p families are responsible for the BFA-inhibited ARF exchange activity found on Golgi membranes, although there is no direct evidence for this conclusion to date.

The first Sec7 domain demonstrated to have ARF exchange activity in vitro was that of the human protein ARNO, which we identified based on sequence similarity to the Gea1/2 proteins in a 200–amino acid region (the Sec7 domain) (Chardin et al., 1996). Surprisingly, the exchange activity of ARNO was found to be resistant to BFA (Chardin et al., 1996). Cytohesin-1 was also found to have ARF exchange activity unaffected by BFA (Meacci et al., 1997). In contrast to the large Gea and Sec7 ARF exchange factors, the ARNO family members are much smaller (approximately 47 kDa), and all contain a C-terminally located PH domain, which mediates membrane binding (Klarlund et al., 1997; Paris et al.,

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1997; Franco et al., 1998). With the entire *Saccharomyces cerevisiae* genome sequenced, we know that no ARNO homolog exists in this organism, suggesting that ARNO function is specific to higher eukaryotes. Hence, there are two classes of Sec7 domain ARF exchange factor: the Gea/Sec7 family of "large" proteins conserved from yeast to humans, and the ARNO family of "small" factors unique to higher eukaryotic cells. To date, all of the large Gea/Sec7 family members tested have BFA-sensitive ARF exchange activity whereas the small exchange factors of the ARNO family have activity resistant to BFA.

The identification of BFA-resistant ARF exchange factors raises questions concerning the determinants of BFA sensitivity. We show here that Gea1/2p and Sec7p are the major essential targets of BFA in vivo in yeast and provide an explanation at the molecular level for the inhibition by BFA of nucleotide exchange on ARF.

Results

The Amount of Gea1p and Sec7p in Cells Is Correlated with Their Level of Resistance to BFA

To determine whether the quantity of Gea1p in cells affects their sensitivity to BFA, strain APY019 *gea1*Δ *gea2*Δ *erg6*Δ containing different *GEA1*-bearing plasmids, each producing a different level of Gea1p, was tested for growth on plates containing different concentrations of BFA (Figure 1A). Since wild-type yeast cells are impermeable to BFA, the *erg6*Δ mutation (which blocks production of ergosterol) must be introduced to allow BFA to enter cells (Graham et al., 1993; Shah and Klausner, 1993; Vogel et al., 1993). The level of Gea1p in APY019 cells carrying plasmids pCLJ90, pAP23, and pCLJ92, respectively, is less than, approximately equal to, and 10-fold higher than the level of Gea1p found in wild-type cells (Figure 1A and data not shown). We tested the ability of APY019/pCLJ90 strains carrying either pAP23, pCLJ92, or no additional source of Gea1p to grow on plates containing different concentrations of BFA. In addition, we tested the same strain overexpressing the nonfunctional *gea1*^{E636K} allele. The corresponding mutation in ARNO (E156K) abolishes exchange activity in vitro (Béraud-Dufour et al., 1998; Cherfils et al., 1998; Mossessova et al., 1998). We observed a direct correlation between the level of ARF exchange–competent Gea1p in cells and their ability to grow in the presence of BFA (Figure 1A). In addition, we observed a synthetic enhancement of protein transport defects caused by limiting amounts of Gea1p and BFA treatment (data not shown).

We tested whether increasing the level of expression of Sec7p in yeast cells would also confer resistance to BFA. A multicopy *SEC7*-bearing plasmid (2μ-*SEC7*) or the corresponding 2μ vector was introduced into strain APY033-9-2 *gea1*Δ *gea2*Δ *erg6*Δ carrying Gea1p at either a low (pCLJ90), normal (pAP23), or high level (pCLJ92) of expression. In each case, cells carrying 2μ-*SEC7* grew significantly better in the presence of BFA than the corresponding cells with the endogenous level of Sec7p (data not shown). We assayed general secretion competence by examining secretion of proteins into

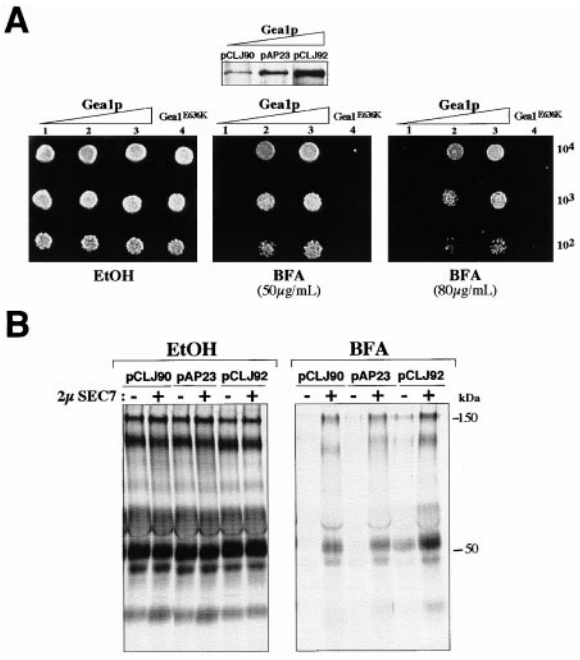

Figure 1. The Quantity of Gea1p and Sec7p Is Correlated with the Level of Resistance to BFA In Vivo

(A) APY019/pCLJ90 was transformed with vector (1), pAP23 (2), pCLJ92 (3), or pCLJ92 E636K (4). Cell suspensions (cell number indicated to the right of the panels) were spotted on plates with EtOH, 50 μg/mL, or 80 μg/mL BFA and incubated for 3 days at 30°C. The level of Gea1p expressed from the different plasmids was monitored by Western blot analysis.

(B) General secretion competence was analyzed in strains APY033-9-2/pCLJ90, APY033-9-2/pAP23, and APY033-9-2/pCLJ92 overexpressing Sec7p in pAP47 (+) or not (−). Cultures were incubated with either BFA (100 μg/mL) or EtOH for 40 min, pulse labeled for 10 min, and chased for 30 min. Proteins secreted into the medium were visualized by SDS-PAGE and fluorography.

the medium (Gaynor and Emr, 1997). Cells were treated with 100 μg/mL (360 μM) BFA for 40 min, then subjected to pulse–chase analysis in the continued presence of BFA. In cells with endogenous levels of Sec7p, secretion was severely inhibited by BFA treatment but was nonetheless correlated with the level of Gea1p present in the cells (Figure 1B). In cells overexpressing Sec7p from a multicopy plasmid, the level of secretion in the presence of BFA was dramatically improved in all three strains tested. These results demonstrate that the levels of both Gea1p and Sec7p in cells are correlated with their level of resistance to BFA. Moreover, the co-overexpression of Gea1p and Sec7p results in a synergistic improvement in the rates of growth and secretion in the presence of BFA.

Strains in which the Sec7 Domain of Gea1p or of Sec7p Is Replaced by that of ARNO Are More Resistant to BFA than Wild-Type Strains

To determine whether the Sec7 domain is involved in sensitivity to BFA in vivo, we constructed Gea1 and Sec7 chimeric proteins in which their Sec7 domains were replaced by that of ARNO (Figure 2A). These chimeric proteins are referred to as G-AR-Gp and S-AR-Sp,

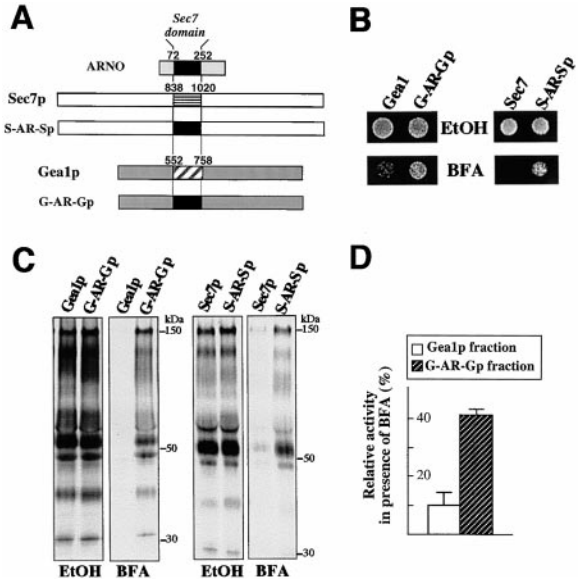

Figure 2. G-AR-G and S-AR-S Chimeric Proteins Are BFA-Resistant Versions of Gea1p and Sec7p

(A) Gea1 and Sec7 chimeric proteins were constructed by replacing the Sec7 domain of the yeast Gea1 and Sec7 proteins by the human ARNO–Sec7 domain. Numbers indicate the first and last amino acid positions of the Sec7 domain of each protein.

(B) Equivalent amounts of APY033-9-2 cells carrying pAP23 (Gea1p) or pAP43 (G-AR-Gp) were spotted on plates with or without BFA (70 μg/mL). Similarly, APY045-18-3 (p90) cells carrying either pAP57 (Sec7p) or pAP58 (S-AR-Sp) were spotted on plates with or without BFA (45 μg/mL).

(C) Strains listed in (B) were incubated with or without BFA (100 μg/mL) for 2 min, pulse labeled for 10 min, and chased for 30 min.

(D) Inhibition of Gea1p or G-AR-Gp chimera-stimulated GTPγS binding to myr-bARF1 by BFA in vitro was monitored. Ni^{2+} Hi-Trap column elution fractions containing Gea1p or G-AR-Gp were tested in the standard GTPγS-binding assay (see Experimental Procedures). Activity was calculated as the rate of binding of [^{35}S]GTPγS to myr-bARF1 stimulated by each fraction. Relative activity in the presence of BFA was calculated as the ratio of activity in the presence of BFA to that in its absence, expressed as a percentage. The mean and standard deviation of three independent experiments are shown.

respectively. Each chimeric protein was able to support vegetative growth and secretion as efficiently as the corresponding wild-type protein (see below). These results were obtained using strains in which the G-AR-Gp chimera was the sole source of Gea1/2p in the cell, and similarly, when S-AR-Sp was the only source of Sec7p in the cell. The G-AR-Gp strain grew significantly better in the presence of BFA than the corresponding strain expressing wild-type Gea1p (Figure 2B). We verified by Western blot analysis that the level of expression of the chimera was equal to that of the wild-type Gea1p in these strains (data not shown). Similarly, a strain expressing the S-AR-Sp chimera conferred a higher level of resistance to BFA than the equivalent strain expressing wild-type Sec7p (Figure 2B).

To monitor the secretion phenotypes of strains expressing either the G-AR-Gp or the S-AR-Sp chimeric proteins as their sole copies of Gea1p and Sec7p, cells were treated with 100 μg/mL BFA for 2 min, then labeled and chased for 30 min. Strains expressing either the G-AR-Gp or the S-AR-Sp chimera had secreted 4- to 5-fold more protein into the medium than the wild-type control after 30 min of chase in the presence of BFA (Figure 2C). Although the chimeric proteins restore the level of secretion significantly in the presence of BFA, the rate of secretion is still reduced compared to non-treated cells (Figure 2C and data not shown).

We assume that the G-AR-Gp and S-AR-Sp chimeras confer resistance to BFA due to an ARF exchange activity resistant to inhibition by BFA. To test this hypothesis, we compared the in vitro exchange activity of wild-type and chimeric versions of Gea1p. Wild-type Gea1p and the G-AR-Gp chimera (each carrying an N-terminal $(His)_6$-tag) were partially purified by Ni-column chromatography. These preparations were tested for their ability to stimulate binding of GTPγS to mammalian recombinant myr-ARF1. In the presence of 300 μM BFA, the ARF exchange activity of the Gea1 protein was 4-fold more sensitive than that of the G-AR-Gp chimera (Figure 2D). Hence, replacing the Gea1p–Sec7 domain with that of ARNO gives rise to a version of Gea1p that has ARF exchange activity in vitro that is much more resistant to BFA than the wild-type protein.

Gea1/2p and Sec7p Are the Major Targets of BFA in Yeast

To determine whether Gea1/2p and Sec7p are the sole essential targets of BFA in the yeast secretory pathway, we constructed a strain in which both Gea1/2p and Sec7p were replaced by the BFA-resistant G-AR-Gp and S-AR-Sp chimeras, respectively. We found that the double-chimera strain grew almost as well on plates containing 100 μg/mL BFA as on the control plates lacking BFA (Figure 3A). To determine whether the resistance to BFA inhibition of secretion in the double-chimera strain was established rapidly after treatment with the drug, we carried out pulse–chase analysis. Following a 2 min preincubation with 100 μg/mL BFA, cells were labeled for 10 min, then protein secretion into the medium was monitored after 0 or 30 min of chase. For the double-chimera strain, the level of secretion compared to that of the untreated control was restored to 60%–75% at 0 min chase (Figures 3B and 3C) and to 75%–93% at 30 min chase (data not shown). Each single-chimera strain secreted medium proteins at a higher rate than the control strain, but as for the growth phenotype, this level was significantly less than for the double-chimera strain (Figures 3B and 3C and data not shown). We also analyzed transport of the vacuolar hydrolase carboxypeptidase Y (CPY) in the double-chimera strain, since transport of CPY is particularly sensitive to BFA treatment (data not shown). The precursor form present in the ER (p1) can be distinguished by size on SDS-polyacrylamide gels from the more highly glycosylated Golgi form (p2) and the smaller mature form (m) produced by cleavage of the N-terminal propeptide in the vacuole. Treatment of cells with 100 μg/mL BFA resulted in a complete inhibition of ER–Golgi transport of CPY in the strain carrying wild-type Gea1p and Sec7p, but for the double-chimera strain, transport of CPY to the vacuole was restored to 84% of the level of untreated cells (Figure 3D). This result is particularly striking since

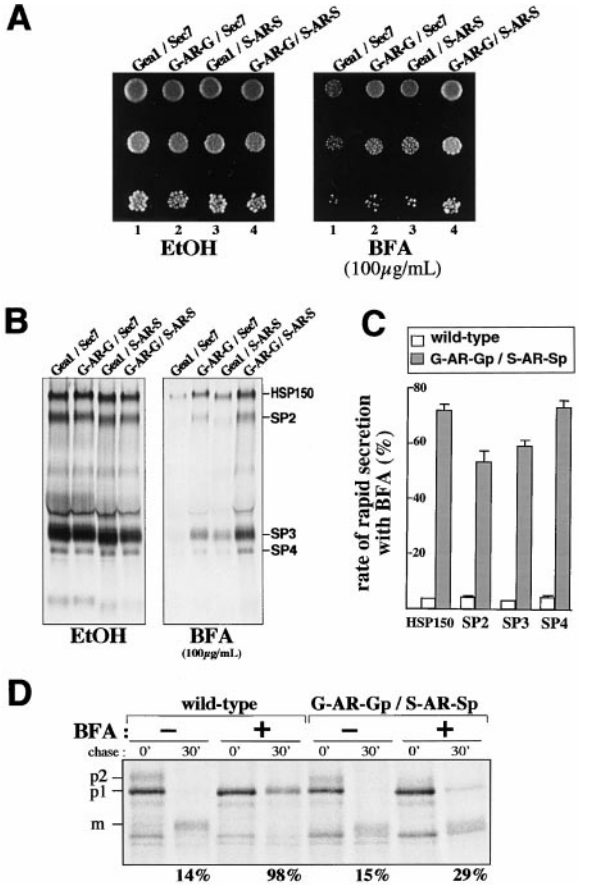

Figure 3. Combined Effects of G-AR-G and S-AR-S Proteins on In Vivo Resistance to BFA

(A) APY045-18-3 cells carrying pAP57(Sec7p)/pAP23(Gea1p) (1), pAP57(Sec7p)/pAP43(G-AR-Gp) (2), pAP58(S-AR-Sp)/pAP23(Gea1p) (3), or pAP58(S-AR-Sp)/pAP43(G-AR-Gp) (4) were spotted on plates with or without BFA (100 μg/mL) and incubated for 3 days at 30°C.

(B) Strains listed in (A) were pulse labeled in presence of BFA (100 μg/mL) as described in Figure 2 (after 0 min chase).

(C) Quantitative comparison of the amount of individual proteins rapidly secreted into the medium in the double-chimera and wild-type strains treated with BFA. The amount of HSP150, secreted protein 2 (SP2), SP3, or SP4 present in the medium at 0 min of chase for BFA-treated strains was quantified by phosphorimager analysis and normalized to that obtained for the corresponding untreated strains. The average and standard error from two independent experiments are shown.

(D) CPY transport and maturation in the wild-type and double-chimera strain with or without BFA were monitored by pulse–chase analysis as described in (B). CPY was recovered by immunoprecipitation. The ER form (p1), Golgi form (p2), and mature vacuolar (m) form of CPY are indicated. Percentages correspond to the proportion of precursor p1 form remaining after 30 min chase, as determined by quantitative phosphorimager analysis.

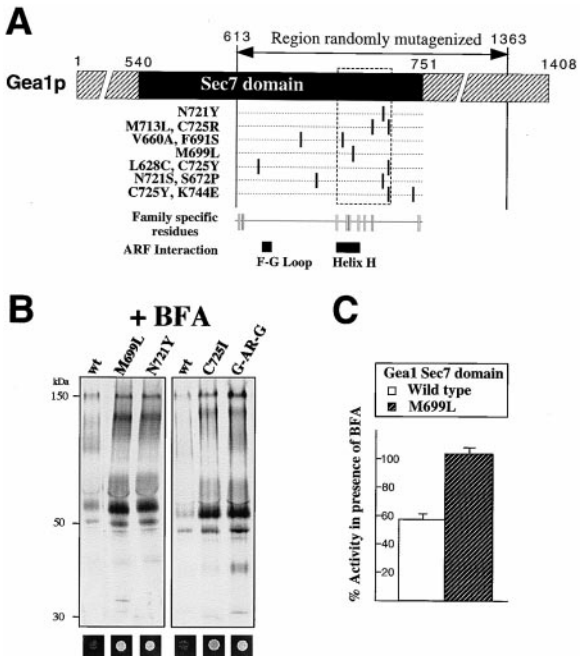

Figure 4. Selection and Characterization of BFA-Resistant Gea1 Mutants: Involvement of a Specific Region of the Sec7 Domain

(A) Schematic drawing of Gea1p and its Sec7 domain, with the region subjected to random mutagenesis and the positions of substitutions in the mutants conferring resistance to BFA indicated. The gray bars represent residues that differ between the Gea/Sec7 (BFA-sensitive) and ARNO (BFA-resistant) families. Note that all mutants contain at least one substitution in a region (boxed) including helix H, one of the two motifs involved in interaction of the Sec7 domain with ARF.

(B) APY033-9-2 (pAP47) cells carrying either pAP23 (Gea1p), p254 (Gea1C725Ip), p255 (Gea1M699Lp), p258 (Gea1N721Yp), or pAP43 (G-AR-Gp) were pulse labeled in the presence of BFA (100 μg/mL) as described in Figure 2 and in parallel were spotted on plates with BFA (100 μg/mL) and incubated at 30°C for 3 days.

(C) The Sec7 domain of yeast Gea1p (wild type) or the same domain carrying the mutation M699L was purified from *E. coli* and tested for stimulation of GTPγS binding to myr-yARF2 in the presence or absence of BFA. For each Gea1–Sec7 domain protein, the quantity of [^{35}S]GTPγS bound to myr-yARF in the presence of BFA was normalized to that obtained for the untreated reaction. Shown are the average and standard error of 10 points from five independent experiments (wild type) and 8 points from four independent experiments (M699L).

in the single-chimera strains, CPY is found primarily in the ER p1 precursor form even after 30 min of chase in the presence of BFA (data not shown).

Isolation of Mutations in *GEA1* Conferring Resistance to BFA

We randomly mutagenized the portion of the *GEA1* gene encoding the region between amino acids 613 and 1363 (which includes the C-terminal 70% of the Sec7 domain), then selected for mutants that grew more quickly than the parental strain in the presence of 50 μg/mL BFA. We obtained seven mutants that reproducibly conferred resistance to BFA in vivo when compared to the wild-type strain (Figure 4A). Strikingly, all seven mutants contained at least one substitution in the 35–amino acid region between residues 691 and 725 (Figure 4A), which overlaps the site of the Sec7 domain involved in interactions with ARF (Cherfils et al., 1998; Goldberg, 1998; Mossessova et al., 1998). No other such "hot spot" was found in the 550–amino acid region of Gea1p subjected to mutagenesis and sequence analysis. Three of the Sec7 domain mutations were of particular interest. In two cases, one single substitution was found in the Sec7 domain: M699L and N721Y, suggesting that in these

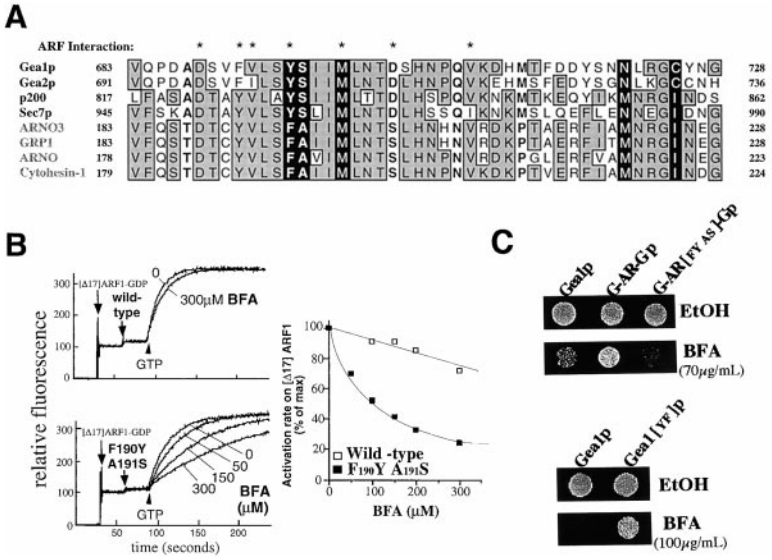

Figure 5. Specific Mutations Can Generate a BFA-Sensitive Form of the ARNO–Sec7 Domain

(A) Alignment of the 40–amino acid region critical for the effect of BFA on the Gea1p–Sec7 domain (boxed region of Figure 4A) with that of both BFA-sensitive (black type) and BFA-resistant (gray type) Sec7 domains. Residues identical in five or more of the proteins shown are shaded, those that differ between BFA-resistant and sensitive families are indicated in bold type, and those important for the effect of BFA are in white on a black backgound. Residues involved in interaction with ARF are indicated (asterisk).

(B) Stimulation of GTP binding to [Δ17]ARF1 by the Sec7 domain of ARNO (wild type) or of ARNO(F190Y A191S) in the presence or absence of BFA was determined by monitoring in real time the tryptophan fluorescence change of ARF1 from the GDP-bound to the GTP-bound state (left panel). At the indicated times, [Δ17]ARF1–GDP (0.5 μM) and ARNO–Sec7 domain (wild type or F190Y A191S) (50 nM) and GTP (100 μM) were added. The rate of acceleration of GTP binding to [Δ17]ARF1 was calculated for the wild-type and mutant ARNO–Sec7 domains and plotted as a function of BFA concentration (right panel). Similar results were obtained using the standard [^{35}S]GTPγS-binding assay (S. Paris, personal communication).

(C) Vegetative growth of APY033-9-2 cells carrying pAP43 (G-AR-Gp), pAP43 FYAS (G-AR F190Y A191S-Gp), or pAP23 (Gea1p) was monitored on plates containing BFA (70 μg/mL) or EtOH after incubation at 30°C for 3 days. Growth of APY033-9-2 cells carrying pAP23 (Gea1p) or p259 (Gea1Y695Fp) in the presence or absence of 100 μg/mL BFA was monitored (right panel).

cases one substitution alone might be responsible for the resistance. Two residues, N721 (again) and C725, were found mutated multiple times (twice and three times, respectively). This region also contains a high frequency of residues that differ between the Gea/Sec7 family of BFA-sensitive ARF exchange factors and the ARNO family of BFA-resistant factors (Figure 4A).

We constructed by site-directed mutagenesis the following Gea1p mutants: Y695F, M699L, N721Y, and C725I. Strains expressing each of these mutants from a low-copy centromeric plasmid as the only source of Gea1p in the cell grew as well as the corresponding wild-type strain under normal conditions. We did not observe a defect in secretion in strains with the M699L, N721Y, or C725I Gea1p mutant as their only source of Gea1/2p (data not shown). In the presence of 100 μg/mL BFA, both growth and secretion were significantly improved compared to the control strain expressing the same level of wild-type Gea1p (Figure 4B). However, none of these single mutants were able to restore secretion to the same level as the G-AR-Gp chimera in the presence of BFA (Figure 4B and data not shown).

To determine whether residues in the Sec7 domain of Gea1p are directly responsible for sensitivity of ARF exchange activity to BFA, we purified the Sec7 domain of Gea1p (either the wild-type or the M699L mutant) from *E. coli*. The Gea1–Sec7 domain proteins stimulated ARF exchange activity in vitro on yeast myr-ARF2 in a concentration-dependent manner, and the M699L mutant was approximately 4-fold less active than the wild-type protein (data not shown). The exchange activity of the wild-type Gea1–Sec7 domain was partially inhibited in the presence of 300 μM BFA (Figure 4C). In contrast, exchange activity of the M699L mutant was not inhibited by this concentration of BFA (Figure 4C). Hence, the M699L mutation in the Gea1p–Sec7 domain gives rise to a version that has BFA-resistant ARF exchange activity in vitro.

The ARNO–Sec7 Domain Containing the Double Substitution F190Y A191S Is Sensitive to BFA In Vitro and In Vivo

The Y695 and S696 residues in Gea1p (corresponding to F190 A191 of ARNO) are among those that differ between the BFA-resistant ARNO family and the BFA-sensitive Gea/Sec7 family, and they fall into the region of the Sec7 domain identified by our random mutagenesis approach as important for the sensitivity of Gea1p to BFA (Figure 5A). To determine whether these residues play a role in the sensitivity of Sec7 domains to BFA, we introduced the double substitution F190Y A191S into the ARNO–Sec7 domain by site-directed mutagenesis. We tested the activity of the mutant protein in stimulating GDP/GTP exchange on [Δ17]ARF1, which lacks the N-terminal amphipathic helix and which is no longer dependent on lipids for nucleotide exchange (Kahn et al., 1992; Paris et al., 1997). Under these conditions, the ARNO(F190Y A191S)–Sec7 domain was almost as active as the wild-type protein in catalyzing exchange (60% of the wild-type level). At 300 μM BFA, activity was inhibited 75% compared to only 20%–30% inhibition for the wild-type ARNO–Sec7 domain (Figure 5B). Hence, substitution of the F190 A191 pair of residues in ARNO for those found in Gea1p (YS) is sufficient to confer BFA sensitivity on the Sec7 domain of ARNO in vitro. Addition of lipid vesicles to the ARNO(F190Y A191S)–[Δ17]ARF1 reaction does not change the level of sensitivity to BFA (S. Paris, personal communication). A very important conclusion of these results is that BFA can act directly on the Sec7 domain or on the Sec7 domain–ARF complex.

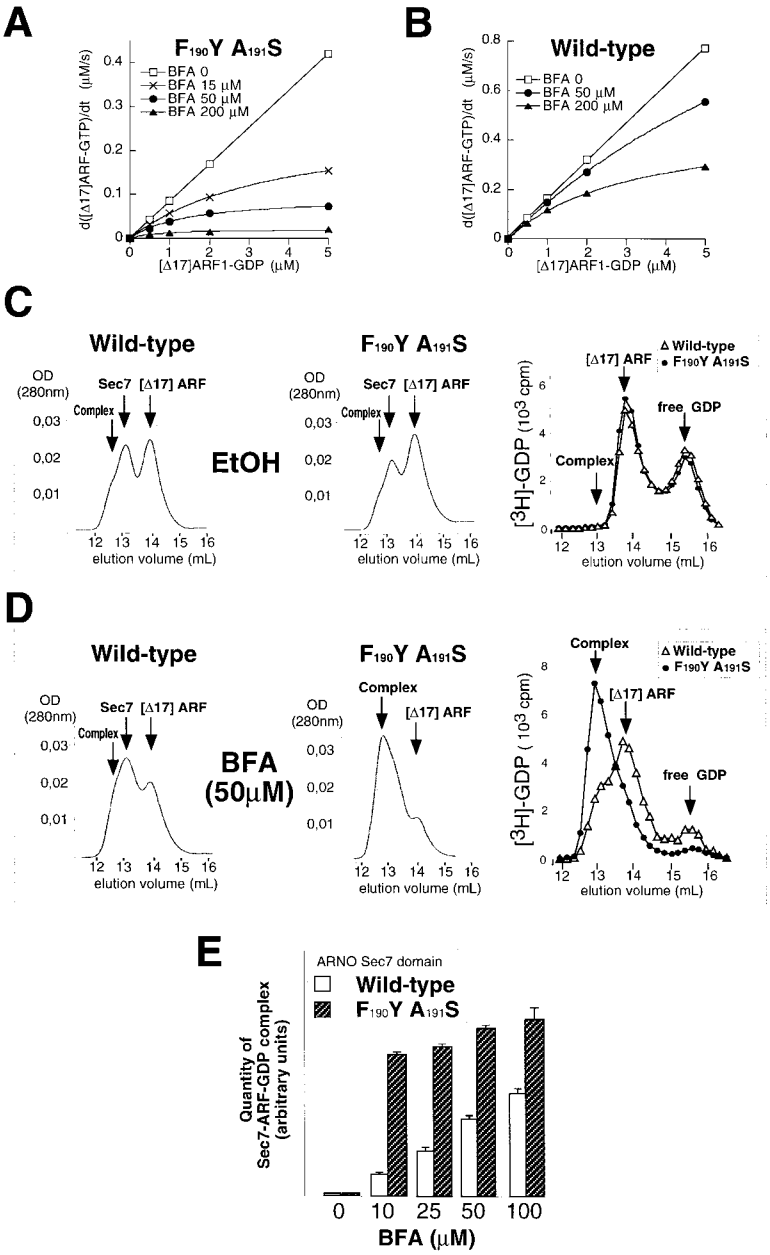

Figure 6. BFA Acts as an Uncompetitive Inhibitor and Stabilizes a [Δ17]ARF1–GDP–ARNO–Sec7 Domain Complex

(A and B) Experiments similar to that shown in Figure 5B were carried out at various concentrations of [Δ17]ARF1–GDP and in the presence of 0.2 μM ARNO(F190Y A191S)–Sec7 domain (A) or wild-type ARNO–Sec7 domain (B). BFA was used at the indicated concentrations. The initial rate of Sec7 domain-catalyzed nucleotide exchange was determined graphically from the fluorescence recordings and plotted as a function of [Δ17]ARF1 concentration. The data were fitted to the Michaelis-Menton equation, $V = k_{cat}[\text{Sec7 domain}][\text{ARF–GDP}]/(K_m + [\text{ARF–GDP}])$.

(C and D) [^{3}H]GDP-labeled [Δ17]ARF1–GDP (10 μM) was mixed with an equimolar quantity of wild-type or ARNO(F190Y A191S)–Sec7 domain and incubated in buffer containing 1 mM Mg^{2+} in the presence of 1% EtOH (C) or of BFA (50 μM) (D). The mixtures were loaded onto a gel filtration column equilibrated with the same buffer. The eluted fractions were analyzed by optical density at 280 nm (left panels), and the amount of [^{3}H]GDP present in each fraction was quantified (right panels). Aliquots of each fraction were analyzed by SDS-PAGE and Coomassie blue staining. The positions of Sec7 domain alone, [Δ17]ARF1 alone, and the [Δ17]ARF1–Sec7 domain complex (determined by SDS-PAGE analysis) are indicated.

(E) To estimate the quantity of ARF–GDP–Sec7 domain complex present for each condition tested, the number of cpm's in those fractions (total 1.8 mL) containing [Δ17]ARF1–Sec7 domain complex but no free [Δ17]ARF1 was determined and normalized to the total number of counts present in fractions eluting from 12 to 17 mL volume.

To determine whether the mutations creating an in vitro BFA-sensitive ARNO–Sec7 domain have an effect on BFA sensitivity of the BFA-resistant G-AR-Gp chimera in vivo, we introduced the double substitution F190Y A191S into the G-AR-Gp chimera. Mutations in the ARNO–Sec7 domain of the chimeric G-AR-Gp have no detectable effect on growth in the absence of BFA, and the level of wild-type and mutant chimeric Gea1 proteins in cells was equivalent as judged by Western blot analysis (data not shown). We tested in parallel the BFA sensitivity of cells carrying the original chimera G-AR-Gp (wild-type ARNO–Sec7 domain), the mutant G-AR(F190Y A191S)-Gp chimera, and wild-type Gea1p. As shown above, yeast cells carrying G-AR-Gp were more resistant to inhibition of growth and secretion than equivalent cells carrying the wild-type Gea1 protein. Strikingly, cells carrying the mutant G-AR(F190Y A191S)-Gp chimera were now as sensitive as those carrying wild-type Gea1p to inhibition of growth by BFA (Figure 5C). Similarly, secretion in the G-AR(F190Y A191S)-Gp chimera cells was inhibited to the same extent as in cells carrying wild-type Gea1p (data not shown). We next tested the reverse substitution (Y695F) in the wild-type Gea1 protein. Consistent with the in vivo results observed for the chimeras, the Y695F single-mutant version of Gea1p was resistant to growth inhibition by BFA (Figure 5C).

BFA Acts as an Uncompetitive Inhibitor that Stabilizes an ARF–GDP–Sec7 Domain Complex

The region identified by mutagenesis as important for the effect of BFA on Sec7 domains in vivo, including

residues Y695 and M699 of Gea1p (F190 and M194 of ARNO), which are also critical determinants of BFA sensitivity in vitro, overlaps the ARF binding site. Hence, the simplest model to explain the difference in sensitivities between different Sec7 domains is that BFA competes with ARF for binding to those that are sensitive. The competition model predicts that increasing the concentration of ARF substrate will lead to a decrease in the level of inhibition of exchange activity by BFA. To test this model, we measured the exchange activity of the Sec7 domain of the F190Y A191S mutant or of wild-type ARNO at different concentrations of [Δ17]ARF1–GDP in the absence or presence of varying concentrations of BFA. Strikingly, in the presence of BFA, increasing the concentration of [Δ17]ARF1–GDP, in fact, increased the inhibitory effect of BFA on the exchange reaction for both the F190Y A191S (Figure 6A) and wild-type ARNO–Sec7 domains (Figure 6B). At 5 μM [Δ17]ARF–GDP, half-maximal inhibition for the F190Y A191S mutant was at ~10 μM instead of ~100 μM for 0.5 μM [Δ17]ARF–GDP (Figure 6A). At all concentrations of [Δ17]ARF1–GDP used, the wild-type ARNO–Sec7 domain was less sensitive to the effects of BFA than the mutant by a factor of approximately 15. In the absence of BFA, the rate of nucleotide exchange increased linearly with the concentration of [Δ17]ARF1–GDP over a 0–5 μM range, indicating that whether mutated (F190Y A191S) or not, the Sec7 domain of ARNO is far from being saturated by its "substrate," [Δ17]ARF1–GDP. However, in the presence of BFA, the rate of GDP/GTP exchange catalyzed by either the wild-type or mutant ARNO–Sec7 domain was clearly saturated for [Δ17]ARF1–GDP in the micromolar range (Figures 6A and 6B). Fitting the data with Michaelis-Menton equations showed that BFA reduced the maximal rate of ARF activation (the "k_{cat}" of the reaction) and the "K_m" for [Δ17]ARF1–GDP by approximately the same factor in a hyperbolic manner (i.e., $K_{m(apparent)} = K_m \cdot K_i/([BFA]+K_i)$). This result is consistent with an uncompetitive inhibition mechanism in which the target of BFA is the enzyme–substrate complex (in this case, an ARF–Sec7 domain intermediate) rather than the enzyme (the Sec7 domain) itself. To further investigate the idea that the target of BFA is a complex between ARF and the Sec7 domain, we tested the ability of BFA to inhibit the exchange reaction when preincubated with either the Sec7 domain alone, [Δ17]ARF1–GDP alone, or the two proteins together. Immediate BFA inhibition of exchange was only observed in the case where [Δ17]ARF1–GDP and the Sec7 domain of ARNO were preincubated together in the presence of BFA before the addition of GTP. If the reaction in the presence of BFA was started by addition of either ARF or the Sec7 domain, the full level of BFA inhibition was only seen after a lag period of a few seconds (data not shown). These results strongly support the conclusion that the target of BFA is a complex between ARF and the Sec7 domain.

To determine the nature of the abortive ARF–ARNO–Sec7 domain complex formed in the presence of BFA, gel filtration experiments were carried out. [Δ17]ARF1–GDP labeled with [^{3}H]GDP was mixed with a stoichiometric amount of ARNO–Sec7 domain (either the F190Y A191S mutant or wild type) and loaded on a Superose column. The column was continuously equilibrated in a running buffer containing 1 mM Mg^{2+} and either supplemented with BFA or not. In the absence of BFA, only a small fraction of [Δ17]ARF1 and ARNO–Sec7 domain were found associated (Figure 6C). The elution profile of [^{3}H]GDP indicated that [Δ17]ARF1 had lost its nucleotide in the complex formed with wild-type or F190Y A191S ARNO–Sec7 domain (Figure 6C). This result is in agreement with previous studies (Paris et al., 1997; Béraud-Dufour et al., 1998). In dramatic contrast to the control without BFA, in the presence of 50 μM BFA, [Δ17]ARF1 and the ARNO(F190Y A191S)–Sec7 domain were found almost completely associated (Figure 6D). Strikingly, [^{3}H]GDP coeluted with this complex (Figure 6D). Hence, BFA stabilizes a complex between the ARNO (F190Y A191S)–Sec7 domain and [Δ17]ARF1–GDP. For the wild-type ARNO–Sec7 domain, 50 μM BFA stabilized the [Δ17]ARF1–GDP–Sec7 domain complex to a lesser extent than for the F190Y A191S mutant (Figure 6D). The amount of ARF–Sec7 domain complex was significantly less and hence the amount of free ARF greater for the wild type than for the mutant as judged from column elution profiles, and the amount of [^{3}H]GDP associated with the complex was approximately 2-fold lower for the wild type (Figure 6D). We repeated these experiments using different concentrations of BFA and quantitated the amount of ARF–GDP in complex with the ARNO–Sec7 domain (Figure 6E). A 10 μM BFA, the amount of ARNO(F190Y A191S)–Sec7 domain–ARF–GDP complex was already close to the maximum, so the increase in the amount of complex up to 100 μM BFA was small. For the wild-type ARNO–Sec7 domain, the amount of Sec7 domain–ARF–GDP complex increased significantly as the concentration of BFA increased from 10 to 100 μM (Figure 6E). Hence, at 10 μM BFA there is six times more Sec7 domain–ARF–GDP complex present for the F190Y A191S mutant Sec7 domain than for the wild type; this difference is less than 2-fold at 100 μM. These results demonstrate that BFA stabilizes an ARF–GDP–Sec7 domain complex and that the ARNO (F190Y A191S) mutant is more sensitive to this effect than the wild-type ARNO–Sec7 domain. Hence, we have shown that BFA inhibits Sec7 domain–catalyzed nucleotide exchange on ARF by an uncompetitive inhibition mechanism in which the reaction intermediate stabilized by BFA is an ARF–GDP–Sec7 domain complex (Figure 7).

Figure 7. Mechanism of Action of BFA
See text for details.

Discussion

The Gea1/2p and Sec7p ARF Exchange Factors Are the Major Essential Targets of BFA in the Yeast Secretory Pathway

In this study, we demonstrate that BFA inhibition of growth and secretion in yeast is largely due to inhibition of the Gea1/2p and Sec7p ARF exchange factors. We

show that the Sec7 domains of Gea1/2p and Sec7p are major factors determining sensitivity to BFA. Proving that a particular protein or set of proteins is the major target of a drug is difficult if not impossible in higher eukaryotic cells. Yeast provides a system in which this type of problem can be addressed, since it is possible to replace the endogenous version of a gene with that of any desired variant and to express the mutant version at the endogenous level. With the entire *Saccharomyces cerevisiae* genome sequenced, we know that Gea1, Gea2, and Sec7 are the only proteins in yeast that contain a highly conserved Sec7 domain. We replaced the Sec7 domains of Gea1p and Sec7p with that of ARNO and introduced the resulting chimeras into a strain devoid of its endogenous copies of Gea1/2p and Sec7p. This double-chimera strain was now almost completely resistant to the inhibitory effects of BFA on growth and secretion. Hence, we can conclude that the nature of the Sec7 domain is the major determinant of BFA sensitivity in the Gea1p and Sec7p ARF exchange factors.

There is a residual level of sensitivity to BFA in the double-chimera strain, which could be due either to the fact that the ARNO chimeras are themselves slightly sensitive to BFA in vivo, or to the fact that other minor BFA targets exist. In support of the former, we show that ARF exchange activity of the partially purified G-ARGp chimera was inhibited 60% by 300 μM BFA in vitro and that at high ARF–GDP substrate concentrations, the Sec7 domain of ARNO is inhibited to a significant extent by BFA. These results demonstrate that the idea of two distinct classes of "BFA-resistant" and "BFA-sensitive" ARF exchange factors is an oversimplification. In addition to Gea1/2p and Sec7p, there are two other ORFs in the *S. cerevisiae* genome with very divergent Sec7 domains (YBL060w and YPR095c). A strain deleted for the former is viable, so it cannot represent an essential target of BFA. Neither protein contains the "FRLPGE" motif, which forms a central part of the ARF binding site and which is invariant in all exchange factors identified to date for the class I and II ARFs (which include yeast ARF1 and ARF2, the only two ARFs that have an essential function in yeast). However, residues that determine the specificity of a given exchange factor for its ARF substrate have not been established, so it does remain a formal possibility that YPR095c encodes a somewhat BFA-sensitive exchange factor for yeast ARF1 or ARF2. Nevertheless, the Sec7 domains of both of these divergent Sec7 domain proteins have F at the position corresponding to F190 of ARNO, which is responsible in large part for its relative resistance to BFA.

The concentrations of BFA that are used for inhibition of growth and secretion of yeast (both here and in previous studies) (Graham et al., 1993; Shah and Klausner, 1993; Vogel et al., 1993) are ~10-fold higher than the highest concentrations used for mammalian cells. This is likely to be due at least in part to differences in permeability of each to BFA. Indeed, wild-type *S. cerevisiae* cells are completely resistant to BFA, so *erg6*Δ mutant strains are used to study the effects of BFA in this organism in order to render them permeable to the drug. In addition, the ARF exchange activity of partially purified Gea1p from yeast was shown to be half-maximally inhibited at 10 μM BFA (Peyroche et al., 1996), a concentration much lower than that administered externally in vivo. It is interesting to note that different mammalian cell lines have quite different sensivities to BFA in vivo (varying over a 500-fold range) (Torii et al., 1995). The reason for this variability is not known. However, in all of these systems as well as in yeast, BFA has similar effects on the structure and functioning of the secretory pathway and its effects are completely reversible. Given the high level of sequence and functional conservation between yeast and mammalian Sec7 domain ARF exchange factors, we predict that the dramatic effects of BFA on the ER–Golgi system of mammalian cells are likely to be a result of inhibition of mammalian homologs of Gea1/2p and Sec7p ARF exchange factors.

Mechanism of Action of BFA

We have shown that BFA acts through a novel and unusual mechanism by stabilizing an ARF–GDP–Sec7 domain protein complex. This complex is the first intermediate on the nucleotide exchange reaction pathway and precedes the formation of the complex with nucleotide-free ARF (Figure 7). This uncompetitive inhibition mechanism could help explain the fact that the concentrations of BFA used to inhibit nucleotide exchange on ARF in vitro vary over approximately two orders of magnitude. The ARF–GDP–Sec7 domain complex recognized by BFA is likely to be a very minor species in vitro, and therefore, the half-maximal inhibitory concentration for a given reaction will be much higher than the true affinity of BFA for its target complex. Although the mechanism of action of BFA provides a major explanation for in vitro differences in sensitivity to the drug, we do not rule out the possibility that other factors may be involved.

In certain mammalian systems, it has been noted that a higher level of BFA sensitivity is observed in vivo compared to in vitro. The BFA concentration required to inhibit COPI binding to Golgi membranes and induce tubule formation in vitro is approximately 10-fold higher than that required for similar effects in vivo (Orci et al., 1991). The half-maximal inhibitory concentration for the ARF exchange activity of purified Golgi membranes from CHO cells was shown to be 10 μM (Donaldson et al., 1992), whereas 0.2–0.4 μM BFA is sufficient to half-maximally inhibit survival of CHO cells (Torii et al., 1995). A possible explanation for these differences is that in vivo, the inhibitory effect of BFA would be favored because the reaction intermediate is present at higher levels than in the corresponding in vitro situation. This could be due to a higher local concentration of exchange factor and ARF–GDP at their site of action in vivo, or to stabilization of the target complex through the action of other proteins or membrane lipids involved in the exchange reaction in vivo. Further work will be required to test these possibilities.

Several residues that are relatively close together in the Sec7 domain crystal structure were found to be critical for BFA sensitivity in vivo and in vitro, including residues M699 and Y695 of Gea1p (M194 and F190 of ARNO). Residue M699 of Gea1p (M194 of ARNO) is strictly conserved among both BFA-resistant and BFA-sensitive families of exchange factors, and yet its mutation to L in Gea1p confers resistance to BFA both in vivo and in vitro. Y695 of Gea1p (F190 of ARNO) differs between the Gea1p/Sec7p and ARNO families and is

Table 1. List of Plasmids Used in This Study

Plasmid	Description
pCLJ90	CEN, *TRP1*, *GEA1*
pCLJ92	2μ, *URA3*, *GEA1*
pCLJ92HT	2μ, *URA3*, *(His)$_6$-GEA1*
pCLJ92 E636K	2μ, *URA3*, *GEA1 E636K*
pAP23	CEN, *URA3*, *GEA1*
pAP32	2μ, *URA3*, *SEC7*
pAP43	CEN, *URA3*, *GEA1-ARNO-GEA1*
pAP43 FYAS	CEN, *URA3*, *GEA1-ARNO (F190Y A191S)-GEA1*
pAP46	2μ, *URA3*, *(His)$_6$-GEA1-ARNO-GEA1*
pAP47	2μ, *LEU2*, *SEC7*
pAP57	CEN, *LEU2*, *SEC7*
pAP58	CEN, *LEU2*, *SEC7-ARNO-SEC7*
p254	CEN, *URA3*, *GEA1 M699L*
p255	CEN, *URA3*, *GEA1 C725I*
p258	CEN, *URA3*, *GEA1 N721Y*
p259	CEN, *URA3*, *GEA1 Y695F*

therefore a critical determinant of the difference in sensitivities of different Sec7 domain proteins to BFA. Introducing the F190Y A191S double substitution into the G-AR-Gp chimera in yeast rendered this BFA-resistant version of Gea1p BFA-sensitive, and the reverse substitution Y695F in Gea1p rendered it BFA-resistant in vivo. Moreover, the F190Y A191S mutant of the ARNO–Sec7 domain was 15-fold more sensitive to BFA inhibition in vitro than the wild-type form. In the recently published structure of the complex between the Gea2–Sec7 domain and nucleotide-free ARF, residues of the Gea2–Sec7 domain corresponding to Y695 and M699 of Gea1p both make several hydrophobic contacts with residues of ARF (Goldberg, 1998). Comparison with the ARF–GDP structure shows that these contacts require extensive rearrangement in the switch I and II regions of ARF. We suggest that BFA affects formation of these contacts, which inhibits transition from an ARF–GDP–Sec7 domain intermediate complex to the nucleotide-free ARF–Sec7 domain complex. Our demonstration that BFA blocks an early step in the exchange reaction for small GTP-binding proteins may provide insight into the general mechanism of nucleotide exchange on small G proteins.

Experimental Procedures

Strains, Plasmids, Media, and Materials

The following yeast strains were used in this study: *APY019 Matα ura3-52 leu2-Δ1 his3-Δ200 lys2-801 ade2-101 trp1-Δ63 gea1::HIS3 gea2::HIS3 erg6Δ/pCLJ90*, *APY033-9-2 MATα ura3-52 leu2 his3Δ200 lys2-801 ade2-101 gea1::HIS3 gea2::HIS3 erg6Δ/pCLJ90*, and *APY045-18-3 ura3-52 leu2 his3Δ200 lys2-801 ade2-101 gea1::HIS3 gea2::HIS3 sec7::KANMX4 erg6Δ/pCLJ90+pAP32*. We used standard yeast genetic techniques and media (Sherman et al., 1979). Plasmids used in this study are listed in Table 1. Details of their construction will be described elsewhere (A. P. and C. L. J., unpublished data). Site-directed mutagenesis was carried out using oligonucleotides carrying the appropriate mutations in standard PCR reactions, followed by cloning into the appropriate vector. BFA was purchased from Alexis Corporation and dissolved in ethanol at 10 mg/mL.

Cell Labeling, Immunoprecipitation, and Western Blot Analysis

Cell labeling, immunoprecipitation, and reimmunoprecipitation with secondary antisera were performed as described (Gaynor and Emr, 1997), except that cells were grown in SD medium supplemented with amino acids and 50 mM Na-HEPES (pH 7) and were labeled with promix-^{35}S (Amersham) at 5 OD/mL in SD medium containing amino acids and 300 μg/mL BSA. To assay media proteins, media fractions were precipitated by the addition of TCA to a final concentration of 7%. After washing the pellet twice in cold acetone, proteins were solubilized in Laemmli sample buffer plus 5% β-mercaptoethanol, boiled, and centrifuged at 13,000 g for 5 min, and 0.25–0.5 OD equivalents were loaded for SDS-PAGE onto a 9% acrylamide gel. Western blot analysis was performed as previously described (Peyroche et al., 1996). Samples were loaded onto 6% SDS-polyacrylamide gels. Gea1 proteins were detected using polyclonal rabbit anti-Gea1 antibodies.

Isolation of BFA-Resistant Mutants

Mutagenic PCR was carried out as described (Peyroche et al., 1996) using oligonucleotides in the *GEA1* sequence flanking the restriction sites BglII and PflMI. Twenty-five colonies were obtained, which grew better than the control on medium containing 50 μg/mL BFA, and plasmids were isolated and sequenced from seven of these clones.

Expression and Purification of Sec7 Domain Proteins

For Gea1p and G-AR-Gp purification, strains CJY052-10-2/pCLJ92HT or CJY052-10-2/pAP46 (1 liter cultures of each) were grown in YPD (yeast extract-peptone-dextrose) to an absorbency at 600 nm of 0.8. The cells were collected and resuspended in 8 mL final volume of buffer A-50 (50 mM NaCl, 20 mM HEPES [pH 7.5], 10% glycerol). Purification was performed as previously described (Peyroche et al., 1996) except that a 1 mL Ni^{2+}-Hi-Trap column (Pharmacia) was used. For purification of the Gea1p–Sec7 domain, pGEA/SEC7/Calc was constructed by insertion of the NcoI/BamHI-digested PCR product encoding the Gea1p–Sec7 domain (amino acids 532–742) into NcoI/BamHI-digested pCALc (Stratagene). pCLJ260 was constructed in the same way, but using Gea1 E544Q E549D M699Lp as a template to generate the Gea1p–Sec7 domain PCR product. Extracts were prepared from *E. coli* strains carrying pGEA/SEC7/Calc or pCLJ260 and passed over a 2 mL calmodulin affinity column. Bound protein was eluted with 1.5 M $(NH_4)_2SO_4$, dialyzed for 1 hr against Tris–HCl 50 mM (pH 8), $MgCl_2$ (1 mM), β-mercaptoethanol (5 mM), then centrifuged for 2.5 hr on a centricon-10 concentrator (Amicon). The Sec7 domain of ARNO (wild type or F190Y A191S) was purified by anion exchange on Q Sepharose (Pharmacia Biotech, Inc.) as described previously (Béraud-Dufour et al., 1998). Purification of [Δ17]ARF1 was performed by gel filtration with Sephacryl S200 HR as described (Antonny et al., 1997).

Expression and Purification of Myristoylated Recombinant Yeast ARF2

Myr-ryARF2 was prepared from *E. coli* coexpressing yeast N-myristoyltransferase and yeast ARF2. Purification was essentially performed as described for myr-bARF1 (Franco et al., 1995) except that the DEAE-Sepharose column was replaced by a Hi-TrapQ column (Pharmacia) and the second chromatographic step was omitted. As judged by Coomassie staining after SDS-PAGE, the ARF preparation was >80% pure after the chromatographic step on the Hi-TrapQ column, and the contamination of myr-ryARF2 by the nonmyristoylated species was <10%. The maximum binding capacity of myr-ryARF2 was determined by measuring the amount of [^{35}S]GTPγS bound at 1 μM free Mg^{2+} and corresponds to approximately 60% of the total amount of myr-ryARF2 present in the reaction. This value is similar to that described for myr-rbARF1 (Franco et al., 1995).

GTPγS Binding Assays

[^{35}S]GTPγS binding to recombinant myristoylated bovine ARF1 (myr-bARF1) was performed as described (Franco et al., 1995) using azolectin vesicles (1.5 gL^{-1}) and 1 mM free Mg^{2+} and with or without 300 μM BFA. All assays contain 1% EtOH, the amount added with 300 μM BFA. The concentration of myr-rbARF1 in each reaction was 1 μM; samples of 25 μL (25 pmol myr-ARF) were removed after 1, 3, and 5 min incubation at 37°C. Binding of [^{35}S]GTPγS to fractions without myr-bARF1 was also monitored. All values were corrected for nonspecific binding of [^{35}S]GTPγS to phospholipids and filters and to protein fractions measured in the absence of myr-bARF1.

GTPγS-binding assays on recombinant myristoylated yeast ARF2 (final concentration 1 μM) were performed at 30°C using azolectin vesicles (0.3–1 mg/mL), 1 mM Mg^{2+}, and either 300 μM BFA or an equivalent volume of EtOH. Samples of 25 μL (25 pmol myr-yARF2) were removed after 40 and 80 s. Preparations of Gea1–Sec7 domain proteins were used in these reactions at a final concentration of 0.25–1 μM (wild type) and 0.5–4 μM (M699L).

Fluorescence Measurements of GDP/GTP Exchange on ARF

The tryptophan fluorescence assay for measuring GTP binding to [Δ17]ARF1 was performed as described (Antonny et al., 1997), at 37°C in 50 mM HEPES (pH 7.5), 100 mM KCl, 1 mM $MgCl_2$, 2 mM DTT supplemented with BFA (0–300 μM) or an equivalent volume of methanol (1% maximum). At the indicated times, [Δ17]ARF1–GDP and ARNO–Sec7 domain (wild type or F190Y A191S) were added, and the reaction was initiated by addition of GTP (100 μM).

Gel Filtration

Gel filtration analysis of [Δ17]ARF1–ARNO–Sec7 domain mixtures was performed as previously described (Béraud-Dufour et al., 1998), except that all experiments were carried out at 4°C in the presence of BFA or an equivalent amount of EtOH.

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